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Glutathione *S*-conjugate transport in hepatocytes entering the cell cycle is preserved by a switch in expression from the apical MRP2 to the basolateral MRP1 transporting protein

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SUMMARY

The multidrug resistance protein MRP1 and its isoform MRP2 are involved in ATP-dependent glutathione *S*-conjugate transport and have similar substrate specificities. MRP2 mediates hepatic organic anion transport into bile. The physiological function of MRP1 in hepatocytes is unknown. Previous results show that MRP1 expression is low in quiescent hepatocytes but increased after SV40 large T antigen immortalization, suggesting a relationship with cell proliferation. Therefore, we determined mrp1 and mrp2 expression in rat hepatocytes in relation to the cell cycle. By varying cell density we obtained cultures that are mainly in G₁ (high density) or have progressed into the S-phase or beyond (low density). In both cultures mrp1 mRNA and protein levels are increased, concomitantly with

the disappearance of mrp2. This switch from mrp2 to mrp1 occurs in the G₁ phase of the cell cycle and is associated with a decreased cell polarity. Mrp1 is located on lateral membranes or on intracellular vesicles, depending on whether cell-cell contact is established. In both locations mrp1 contributes to cellular glutathione *S*-conjugate efflux and protects against oxidative stress-inducing quinones. We conclude that a switch in expression from the apically located mrp2 to the basolaterally located mrp1 preserves glutathione *S*-conjugate transport in hepatocytes entering the cell cycle and protects against certain cytotoxic agents.

Key words: Multidrug resistance protein, Glutathione *S*-conjugate transport, Hepatocyte, Localization, Cell cycle

INTRODUCTION

The multidrug resistance protein MRP (also called MRP1) was discovered as a protein associated with multidrug resistance in cancer cells. The *MRP* gene was isolated by Cole et al. (1992) from a doxorubicin-resistant small cell lung cancer cell line that exhibits non-P-glycoprotein-mediated multidrug resistance. MRP is an 1531-amino acid, 190 kDa, N-glycosylated integral membrane protein, encoded by a 6.5 kb mRNA (Cole et al., 1992; Almquist et al., 1995). It has been demonstrated that MRP transports organic anions, mainly GS-conjugates, in an ATP-dependent fashion (Müller et al., 1994; Leier et al., 1994; Jedlitschky et al., 1994). Substrates include, oxidized glutathione (GSSG) (Leier et al., 1996) and possibly reduced glutathione (GSH) (Rappa et al., 1997), and a whole range of glutathione *S*-conjugate (GS-conjugates) (for reviews see Müller and Jansen, 1997; Müller et al., 1996; Lautier et al., 1996; Loe et al., 1996). In the liver, mrp1 is mainly expressed in bile duct epithelial cells and located on basolateral membranes (Roelofsen et al., 1997a). Due to its low expression, mrp1 localization could not be determined in hepatocytes (Roelofsen et al., 1997b). However, in hepatocyte-

derived cells such as HepG2 hepatoma cells and SV40-immortalized human hepatocytes, MRP1 is located basolaterally (Roelofsen et al., 1997b). A homologue of mrp (mrp2) was cloned from rat liver (Büchler et al., 1996; Paulusma et al., 1996) and turned out to be identical to the canalicular multispecific organic anion transporter (cmoat), which is involved in the transport of organic anions into bile. Mrp2 displays a similar substrate specificity as the original mrp. The absence of mrp2 in the mutant TR⁻ rat (Büchler et al., 1996; Paulusma et al., 1996) explains the deficiency in the biliary organic anions transport in this strain (Oude Elferink and Jansen, 1994). Mrp2 is localized exclusively to the canalicular membrane domain (Büchler et al., 1996; Paulusma et al., 1996). Recently, additional members of the human MRP family were discovered (MRP3 to MRP6) with different tissue distributions (Kool et al., 1997). The substrate specificities of these MRP isoforms have not yet been determined.

While the substrate specificity of mrp1 and mrp2 is similar if not identical, their expression in hepatocytes is different. The finding that mrp1 expression is up-regulated in hepatoma cells and SV40-immortalized hepatocytes (Roelofsen et al., 1997b), suggests that mrp1 expression and function may be associated

with cell proliferation. Therefore, we studied the expression and localization of these two mrp isoforms in hepatocytes in relation to the cell cycle.

Several groups have shown that the disruption of cell-cell contacts during the isolation of hepatocytes induces the expression of immediate early genes such as *c-myc*, *c-fos* and *c-jun*, which indicates entry into the cell cycle (Loyer et al., 1996; Kumatori et al., 1991; Shimbara et al., 1992). Also the progression through the cell cycle depends on cell-cell contact. Cells plated at low density will enter the S-phase while cells plated at high density do not and display a more differentiated phenotype (Kumatori et al., 1991; Shimbara et al., 1992; Ben-Ze'ev et al., 1988; Nakamura et al., 1983). Loyer et al. (1996) defined a restriction point (R-point) in late G₁ phase. Hepatocytes in high density cultures do not cross this R-point without the help of growth factors. Crossing the R-point gives rise to cyclin D1 induction and subsequent entry in the S-phase. We made use of this cell density-dependent progression through the cell cycle to obtain cell cultures that are mainly in G₁ (high density) or beyond G₁ (low density). Furthermore, the different cell densities were useful in comparing the localization of mrp1 and mrp2 in relation to cell-cell contact and cell polarization.

The current study shows that mrp1 is induced in both low and high density hepatocyte cultures. In contrast, mrp2 is down-regulated in these cultures. The switch in the expression of mrp2 to mrp1 occurs in the G₁ phase of the cell cycle and preserves GS-conjugate transport across the plasma membrane in cells entering the S-phase. These results indicate that while mrp2 has an important function in the biliary transport of organic anions in quiescent hepatocytes, mrp1 takes over when hepatocytes enter the cell cycle and provides protection against oxidative stress.

MATERIALS AND METHODS

Hepatocyte isolation and culture

Normal male Wistar rats, weighing approx. 250 g, were obtained from Harlan-CPB, Zeist, The Netherlands. Male TR⁻ mutant rats of the same weight came from our own breeding colonies. They were kept under routine laboratory conditions at the Central Animal Laboratory of the University of Groningen. Animals received standard laboratory chow and had free access to food and water. Hepatocytes were isolated by the method of Berry and Friend (1969) and their viability was \pm 90%. Hepatocytes were suspended in Williams' medium E containing: penicillin (100 i.u./ml)/streptomycin (100 U/ml), 10% fetal calf serum (all from Gibco, Breda, The Netherlands), 20 mU/ml insulin (Novo Nordisk, Bagsvaerd, Denmark) and 50 nM dexamethasone (Sigma, St Louis, MO). Cells were plated on plastic culture dishes (Costar, Badhoevedorp, The Netherlands) and glass coverslips at low (1.4×10^4 cells/cm²) and high (11.1×10^4 cells/cm²) cell density. After 4 hours the media were replaced with the same media described above without dexamethasone. Cells were cultured for up to 4 days. Upon harvesting, one dish at each time point was trypsinized and the cell number was determined by counting in a Bürker-Türk chamber.

Analysis of mRNA by RT-PCR

Total RNA was isolated from freshly isolated and cultured hepatocytes using TRIzol Reagent (Gibco Laboratories, Grand Island, NY) according to the manufacturer's instructions. Subsequently, mRNA was isolated using the Oligotex mRNA mini-kit (Qiagen GmbH,

Hilden, Germany). RT-PCR was performed as described previously (Vos et al., 1998). Glyceraldehyde 3-phosphate dehydrogenase (gapdh) was used as an internal control in each PCR-reaction. The number of cycles were 20 for gapdh, 22 for mrp2, 25 for mrp1b, and 27 for mrp1. Sense and anti-sense primer sequences used were: mrp1, 5'-TTCTAGTGTGGACGAGGCT-3' and 5'-TGGCCATGCTATGAAGACG-3' (Mayer et al., 1995); mrp2, 5'-ACCTCCACGTAGTGATCCT-3' and 5'-GATTTCCCAGAGCCTACAGT-3' (Paulusma et al., 1996); mrp1b, 5'-GAAATAATGCTTATGAATCCCCAAAG-3' and 5'-GGTTTCATGGTCGTCGTCTCTTGA-3' (Zhang et al., 1996); gapdh, 5'-CCATCACCATCTTCCAGGAG-3' and 5'-CCTGCTTACCACCTTCTTG-3' (Fort et al., 1985), respectively. The resulting PCR-products are shown in Fig. 2. Sequence analysis of the mrp1 PCR-product confirmed the specificity of the PCR-primers for mrp1. In each experiment, water was used as a negative control. 10 µl of PCR product was loaded on a 2.5% agarose gel and stained with ethidium bromide. Images were taken using an Image Master VDS (Pharmacia Biotech, San Francisco, CA).

Immunoblot analysis

Cultured cells were washed twice with HBSS without Ca²⁺ and Mg²⁺ (Gibco, Breda, The Netherlands) and harvested from the culture flasks using a rubber policeman. Cells were permeabilized by freezing (-180°C) and rapid thawing (37°C) in the presence of a cocktail of protease inhibitors (1:10, w/v) in 5 mM Hepes/KOH (pH 7.4) (Complete, from Boehringer Mannheim (Almere, The Netherlands)). The cell homogenates were centrifuged (15 minutes, 5000 g) and the resulting pellets were dissolved in sample buffer (5 µg/µl protein) and used as crude membrane fractions. The supernatant was treated with 10% perchloric acid to precipitate cytosolic proteins, and centrifuged (15 minutes, 5000 g). The pellets were dissolved in sample buffer (5 µg/µl protein) and used as cytosolic fractions. Equal amounts of membrane or cytosolic proteins were size fractionated on 7.5% or 10% SDS/polyacrylamide gels. Thereafter, proteins were transferred to nitrocellulose and incubated with the primary antibody, followed by a peroxidase-labeled secondary antibody. For detection the enhanced chemiluminescence kit from Amersham (Den Bosch, the Netherlands) was used.

Immunofluorescence microscopy

For immunolocalization of mrp1 and mrp2, cells grown on coverslips were fixed for 10 minutes in acetone (-20°C), rehydrated in PBS, and incubated overnight with the primary antibody diluted in 1% BSA in PBS at 10°C . Cells were then washed, incubated with the secondary FITC-labeled antibody diluted in 1% BSA in PBS, washed again and mounted in Prolong Antifade mounting medium (Molecular Probes Europe, Leiden, The Netherlands). Control stainings were performed by substituting the primary antibody with preimmune serum and showed no significant staining. Images were taken with a confocal scanning laser microscope (CSLM) (TCS 4D, Leica Heidelberg, Germany) equipped with an argon/krypton laser and coupled to a Leitz DM IRB (Leica Heidelberg, Germany) inverted microscope.

The percentage of cells in the S-phase was assessed following the protocol for the bromodeoxyuridine (BrdU) labeling and detection kit I from Boehringer Mannheim (Almere, The Netherlands). Briefly, hepatocytes grown on glass coverslips at low and high density, were incubated with 10 µM BrdU for 6 hours. Thereafter, cells were fixed in 70% ethanol (in 50 mM glycine buffer, pH 2.0), incubated with a monoclonal directed against BrdU followed by a FITC-labeled secondary antibody. Cells were counterstained with propidium iodide. Representative images in the red (propidium iodide) and the green (FITC) channel were taken with a CSLM. Total cell number (low density: \pm 100 cells and high-density \pm 300 cells per time point) and BrdU-positive cells were counted. From these values, the percentage of BrdU-positive cells for each time point and cell density was calculated from three identical experiments.

Detection and measurement of transport activity

For measurement of mrp-mediated transport out of cells, the fluorescent glutathione-methylfluorescein (GS-MF) was used as described previously (Roelofsen et al., 1997b, 1998). GS-MF is formed intracellularly from the nonfluorescent chloromethylfluoresceindiacetate (CMFDA) (Molecular Probes Europe, Leiden, The Netherlands) by the subsequent action of esterase and glutathione S-transferase. CMFDA is taken up by diffusion. GS-MF is not excreted into bile of the mutant TR⁻ rat indicating that it is a substrate for mrp2 (Roelofsen et al., 1998). Hepatocytes cultured in 9 cm² dishes (3 dishes for each time point), were loaded with CMFDA (10 nmol/10⁶ cells) for 60 minutes at 10°C. Thereafter cells were washed once with ice-cold Hepes-buffered medium (130 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1.3 mM CaCl₂, 1.2 mM KH₂PO₄, 19.7 mM Hepes and 5 mM D-Glucose, pH 7.4) and stored on ice. The initial GS-MF efflux rate was determined by adding medium of 37°C. Every 30 seconds a 200 µl sample was removed for up to 2.5 minutes. Fluorescence was measured with a fluorescence ELISA-plate reader (Titertek Fluoroskan II, ICN Biomedicals, Zoetermeer, The Netherlands) (excitation: 490 nm; emission: 520 nm). Fluorescence was quantified by correlating it to a calibration curve (0.1–1.0 µM GS-MF) in the linear range, as described by Roelofsen et al. (1997b).

Detection of intracellular mrp activity was performed as described previously (Roelofsen et al., 1997b). Briefly, cells grown on coverslips were incubated with CMFDA (2.5 nmol/10⁶ cells) for 15 minutes at 37°C. Coverslips were transferred to Hepes-buffered medium and stored on ice. The intracellular accumulation of the substrate was visualized by confocal microscopy.

MTT test

Hepatocytes isolated from normal rats were cultured for 3 days at low density in 96 well plates. Thereafter, cells were incubated with various concentrations of menadione (0–20 µM), tertiar-butyl-hydroquinone (TBHQ; 0–300 µM) or hydrogen peroxide (0–800 µM), in the absence or presence of 25 µM of the specific MRP inhibitor MK571 (kindly provided by Dr A. W. Ford Hutchinson, Merck-Frosst, Pointe Claire-Dorval, Quebec, Canada). After 20 hours, cell death was determined by MTT test. The cells were incubated with 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; from Sigma, St Louis) for 3.45 hours. The media were removed and the blue product, formed by living cells, was dissolved in DMSO and quantified by measuring the absorbance at 540 nm with background subtraction at 690 nm. The concentrations at which 50% of the cells had died (LD50) were calculated. The test was performed on cells from 3 separate isolations. Significant differences obtained in the absence and presence of MK571 were determined using a two-sided paired Student *t*-test.

Antibodies

The rabbit polyclonal antibody against MRP1 (MRPk5) was raised against the peptide RGLFYsMAKDAGLV located at the carboxyl terminal of human MRP1 (Roelofsen et al., 1997b). The antibody reacts with human and rat MRP1/mrp1 but not with MRP2/mrp2 or MRP3/mrp3 since no signal could be detected on western blots of membrane preparations of human liver (Roelofsen et al., 1997b), which expresses high levels of MRP2 and MRP3 (Kool et al., 1997). Furthermore, equal amounts of mrp were detected in control and TR⁻ hepatocytes (Roelofsen et al., 1997b), while mrp3 has been shown to be upregulated in rats with a similar genetic defect (Kiuchi et al., 1998; Hirohashi et al., 1998). The polyclonal anti-mrp2 (MRPk4) was raised in rabbits against a peptide comprising the last 15 amino acids of the carboxyl end of rat mrp2 (AKEAGIENVNHTL) (Vos et al., 1998). On western blot the mrp2 antibody specifically recognizes a 190 kDa protein in normal liver. This protein is absent in membrane fractions from mrp2-deficient (TR⁻) rats. The rabbit anti-c-myc and the mouse monoclonal anti-cyclin D1 antibodies were from Santa Cruz Biotechnology (Heidelberg, Germany). FITC-labeled sheep

anti-rabbit IgG was from Sigma (St Louis MO). Horseradish peroxidase labeled swine anti-rabbit IgG and goat anti-mouse IgG were from DAKO (Glostrup, Denmark).

RESULTS

Manipulation of the hepatocyte cell cycle by altering cell density

Hepatocytes were cultured for up to 96 hours after seeding in two densities: 1.4 × 10⁴ cells/cm² and 11.1 × 10⁴ cells/cm². The expression of c-Myc (early G₁) and cyclin D1 (late G₁) was determined by western blotting (Fig. 1A). c-Myc is detected as a double band of 67 and 63 kDa. The 67 kDa protein is the most abundant. Hann et al. (1988) showed that these proteins are derived from different splicing products. c-Myc is already expressed in freshly isolated hepatocytes (0 hours), indicating a rapid entry into the cell cycle after isolation. c-Myc is found at all time points for the two culture conditions. In contrast, cyclin D1 is expressed only in low density cultures, starting at 48 hours and increases further over the following 2 days of culture. In addition, DNA-synthesis was measured via the incorporation of the thymidine analogue BrdU to determine the proportion of cells in the S-phase. Fig. 1B shows that, during the 6 hour incubation period with BrdU, maximally 26.5 ± 5% of the hepatocytes in low density cultures and 6.1 ± 0.7% in high density cultures are in the S-phase at 48 hours and 96 hours, respectively. These data indicate that hepatocytes in both culture conditions have entered the G₁ phase of the cell cycle (c-myc expression) but low density cultured hepatocytes have progressed to late G₁ (cyclin D1 expression) and beyond (BrdU incorporation) while cells in high density cultures remain predominantly in G₁.

Induction of mrp1 and down-regulation of mrp2 in cultured hepatocytes

Fig. 2 shows the mRNA expression, determined by RT-PCR, of *mrp1*, *mrp2* and the multidrug resistance gene *mdr1b*, in both low and high density cultured hepatocytes. *Mdr1b* expression was determined for comparison. The rat *mdr1b* is involved in the ATP-dependend transport of hydrophobic, mostly basic, compounds. In line with previous reports (Hirsch-Ernst et al., 1995; Lee et al., 1993; Fardel et al., 1992), *mdr1b* is strongly induced within 24 hours in both high and low density cultures and remains at this level. Up-regulation of *mrp1* mRNA is found in both low and high density cultured cells. In low density cultures maximal expression is reached at 24 hours and remains at this level. In high density cultures a small increase in *mrp1* expression at 24 hours is observed, followed by a further increase at 96 hours of culture. In contrast, *mrp2* mRNA rapidly decreases in low density cultures and has almost disappeared at 72 hours. In high density cultures a small decrease in *mrp2* expression between 0 and 24 hours is observed. From 24 to 72 hours *mrp2* levels remain unchanged. At 96 hours levels are further decreased, reaching a similar level to that found in low density cultures.

Mrp2 protein is abundant in freshly isolated hepatocytes (Fig. 3, 0 hours). At 24 hours, levels of mrp2 in both cultures are comparable with that in freshly isolated cells. In low density cultures mrp2 expression starts to decline at 48 hours and is no longer detectable at 72 hours. In high density cultures,

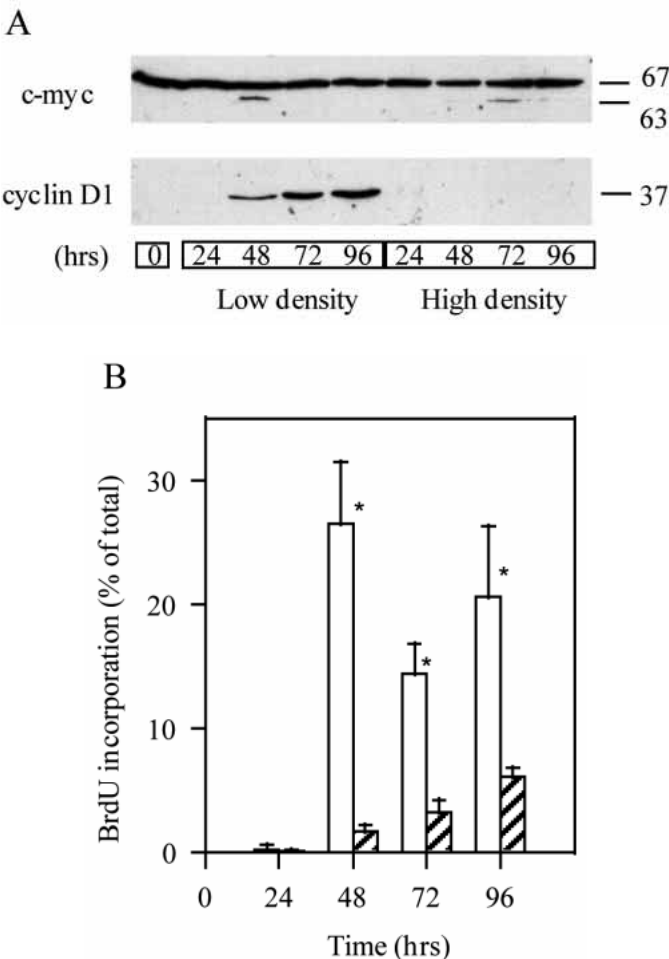


Fig. 1. Characterization of low and high density hepatocyte cultures by the detection of c-myc, cyclin D1 and DNA synthesis. (A) Western blotting. Cytosolic fractions, prepared from homogenates of freshly isolated hepatocytes (0 hours) and hepatocytes cultured for 24, 48, 72 and 96 hours at low and high cell density, were subjected to SDS-PAGE followed by immunoblotting. Protein levels of c-myc and cyclin D1 were detected. The migration positions of these proteins (kDa) are indicated. c-Myc is detected as a doublet of 67 and 63 kDa. Cyclin D1 was found at 37 kDa. (B) BrdU incorporation. The percentage of cells that are in the S-phase was determined by growing hepatocytes on glass coverslips for 24, 48, 72 and 96 hours at low and high density and incubated them with 10 μ M BrdU for 6 hours. Thereafter, cells were stained with a monoclonal against BrdU followed by a FITC-labeled secondary antibody and counterstained with propidium iodide. Representative images were taken and total cell number and BrdU-positive cells were determined from three identical experiments. The percentage of BrdU-positive cells for each time point and cell density was calculated. Low density: white bars, high density: hatched bars. * P <0.01, determined by unpaired two-sided Student's t -test.

mrp2 protein is strongly decreased but is still detectable at 96 hours. In contrast, mrp1 protein is not detectable under these conditions in freshly isolated cells and in cells cultured for 24 hours but is induced between 24 and 48 hours in low density cultures and at 96 hours in high density cultures. These experiments indicate that cell density influences the expression of mrp1 and mrp2. Furthermore, it is striking that the up-

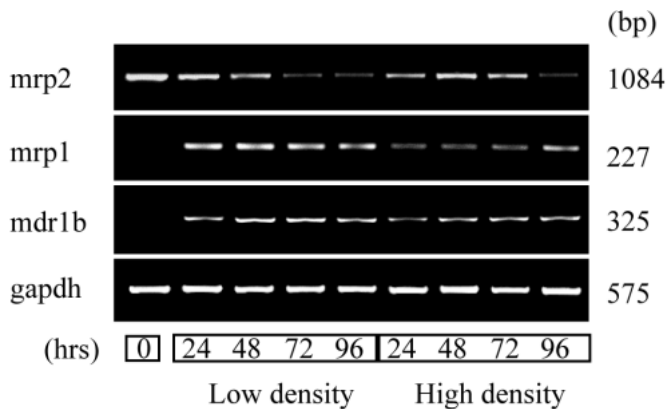


Fig. 2. The mRNA expression of *mrp1*, *mrp2* and *mdr1b* in hepatocytes cultured at low and high density. The relative mRNA levels of *mrp1*, *mrp2*, *mdr1b* and *gapdh* in freshly isolated hepatocytes (0 hours) and hepatocytes cultured for 24, 48, 72 and 96 hours at low and high cell density were determined by RT-PCR with specific primers. The PCR-products were separated on an agarose gel. The corresponding basepair length of the PCR-products is indicated.

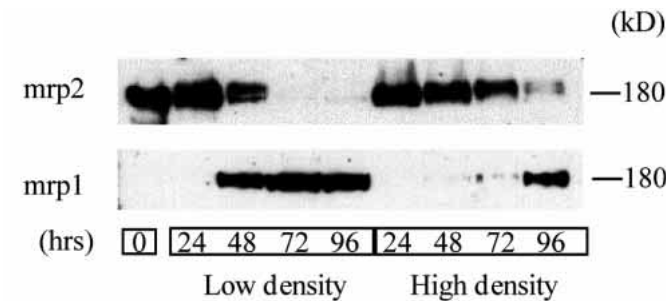


Fig. 3. Immunoblot analysis of mrp1 and mrp2 levels in hepatocytes cultured at low and high density. Crude membrane fractions, prepared from homogenates of freshly isolated hepatocytes (0 hours) and hepatocytes cultured for 24, 48, 72 and 96 hours at low and high cell density, were subjected to SDS-PAGE followed by immunoblotting. Mrp1 and mrp2 were detected with the MRPk5 and MRPk4 polyclonal antibodies, respectively. The migration positions of mrp1 and mrp2 (180 kDa) are indicated.

regulation of mrp1 and the down-regulation of mrp2 appear to be closely coupled.

When these results are compared with the expression pattern of cell cycle markers in Fig. 1 it can be concluded that the reciprocal change in mrp1 and mrp2 expression is induced before the expression of cyclin D1 in the G₁ phase of the cell cycle.

Induction of mrp1 correlates with increased plasma membrane transport activity

The transport activity of mrp1 was studied in TR⁻ rat hepatocytes. These rats lack the mrp2 protein (Büchler et al., 1996; Paulusma et al., 1996). Cells were cultured for up to four days at low and high density. Each 24 hours, the efflux of the fluorescent mrp substrate GS-MF was measured and the mrp1 protein expression was determined. The pattern of mrp1 induction in TR⁻ hepatocytes (Fig. 4A) is similar to that in

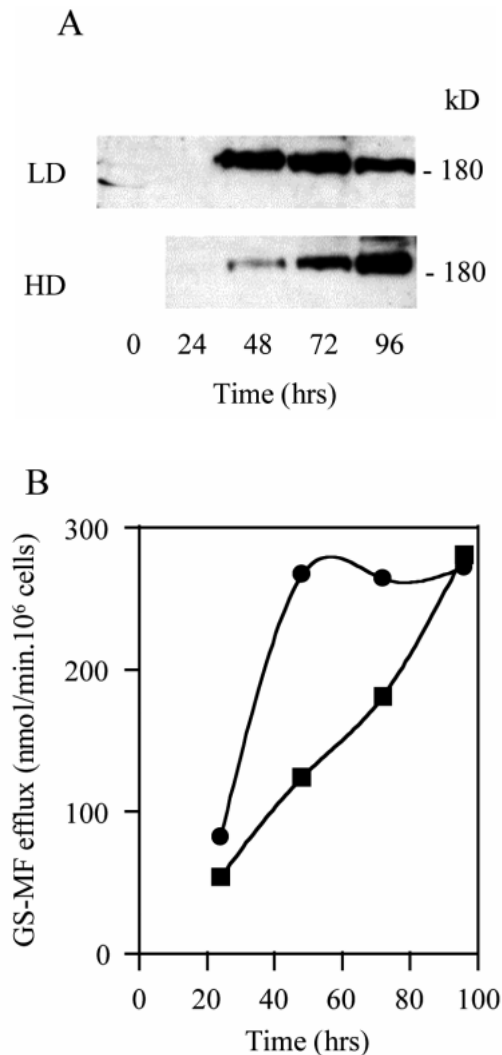


Fig. 4. Comparison of mrp1 protein expression and transport activity in TR⁻ hepatocytes. Experiments were performed with TR⁻ hepatocytes that do not express the mrp2 protein (A) Western blotting. Crude membrane fractions, prepared from homogenates of freshly isolated TR⁻ hepatocytes (0 hours) and TR⁻ hepatocytes cultured for 24, 48, 72 and 96 hours at low and high cell density, were subjected to SDS-PAGE followed by immunoblot. Mrp1 was detected with the MRPk5 polyclonal antibody. The migration position of mrp1 (180 kDa) is indicated. (B) GS-MF efflux. TR⁻ hepatocytes cultured for 24, 48, 72 and 96 hours at low (●) and high (■) cell density were loaded with CMFDA at 10°C. The initial efflux rate at 37°C of the intracellular formed fluorescent GS-conjugate GS-MF was determined.

normal hepatocytes described above (Fig. 2). Fig. 4B shows that hepatocytes cultured at low density display a more than 3-fold increase (from 82 to 272 pmol/minute/10⁶ cells) of GS-MF efflux between 24 and 48 hours in culture. Thereafter transport activity remains at this higher level. Hepatocytes cultured at high density, show a more gradual increase in transport activity. At day 4, transport activity in high density cells has reached a similar level to that in low density cultures. The pattern of mrp1 induction correlates well with the observed increase in transport activity for both high and low density cultures (compare Fig. 4A and B). These data suggest

that mrp1 is responsible for the increase in GS-conjugate transport activity.

Different localization of mrp1 and mrp2 in low and high density cultures

The subcellular localization of mrp1 was studied in low and high density cultured hepatocytes (Fig. 5). In cells cultured for 48 to 96 hours at low density, mrp1 is mainly localized to intracellular punctate structures, most likely representing vesicles (Fig. 5A). No labeling of the plasma membrane is detectable. However, some cells that are in contact with neighboring cells show accumulation of mrp1 on lateral membranes (see arrows, Fig. 5B). The abundance of mrp1-positive vesicular structures appears inversely related to the amount of mrp1 on the lateral membrane: when lateral staining is absent, vesicular staining is prominent and when lateral staining is present, vesicular staining is strongly reduced or absent (compare e.g. Fig. 5A and B). In high density cultured hepatocytes cultured for 96 hours, mrp1 is mainly present in lateral membranes and not in vesicles (Fig. 5C). These data indicate that in the absence of cell-cell contact, mrp1 is present in intracellular vesicles.

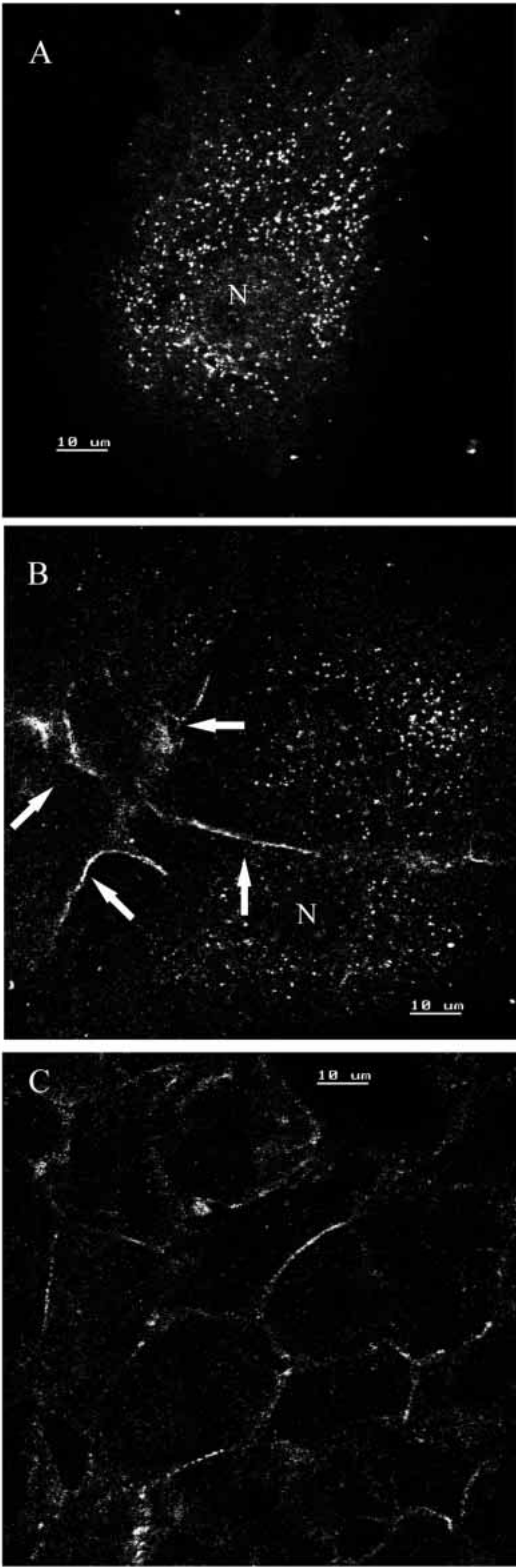
The localization of mrp2 is shown in Fig. 6. In hepatocytes cultured at low density for 24 hours, mrp2 is mainly located to vesicular structures (A). Occasionally, accumulation of mrp2 on the membrane of large apical vacuoles formed between two cells can be observed (see arrow). In high density cultured cells mrp2 is mainly localized to apical structures (B). The mrp2-positive apical vacuoles disappear gradually with prolonged culture times and are not observed after 72 hours in culture (not shown).

To determine whether mrp1 mediates the accumulation of substrate into the vesicular lumen, vesicular accumulation of the fluorescent GS-MF was visualized by confocal microscopy in cells cultured for 72 hours at low density. For this experiment TR⁻ hepatocytes were used to exclude possible vesicular uptake via mrp2. As shown in Fig. 7A, numerous fluorescent punctate structures could be identified, indicating that the substrate has been transported into the vesicular lumina.

In identical experiments performed with normal hepatocytes, cultured for 24 hours, GS-MF was taken up in vesicular structures in low density cultured hepatocytes, and in apical vacuoles in hepatocytes cultured at high density (Fig. 7B and C, respectively). Since mrp1 protein is not detectable in both low and high density cultured hepatocytes at this time point (see Fig. 3), the observed transport of GS-MF into intracellular structures is likely to be mediated by mrp2.

Protective function of mrp1 against agents that induce oxidative stress

To learn more about the role of mrp1 in hepatocytes that have entered the cell cycle we determined whether mrp1 could protect against oxidative stress. Hepatocytes isolated from normal rats were cultured for 3 days at low density. Under this condition mrp1 is maximally induced while mrp2 is not detectable (Figs 2 and 3). The effect on cell death of various concentrations of menadione (0-20 μ M), TBHQ (0-300 μ M) or hydrogen peroxide (0-800 μ M) was tested in the absence or presence of 25 μ M of the specific MRP inhibitor MK571 (Gekeler et al., 1995). This concentration of MK571 was found



to inhibit GS-MF efflux by 60% (not shown) and was not toxic. Differences in cell death were determined by MTT test. Results (Table 1) show that the concentrations of menadione and TBHQ at which 50% of the cells die (LD50) decrease 3-fold when mrp1 is inhibited (+MK571). Inhibition of mrp1 did not influence the LD50 for H₂O₂. These data indicate that mrp1

Fig. 5. Different localization of mrp1 in low and high density cultures. Hepatocytes cultured for 72 hours at low density and 96 hours at high density were stained for mrp1 with the polyclonal antibody MRPk5 followed by a secondary FITC-labeled antibody. Images were taken with a confocal scanning laser microscope. (A) In low density cultures, mrp1 is located to intracellular vesicles in cells that have not established cell-cell contact. No staining of the plasma membrane was observed. (B) In cells that do establish cell-cell contact in low density cultures, mrp1 staining is found on lateral membranes (see arrows). This is accompanied by a reduction in the number of mrp1-positive vesicular structures (compare A and B). (C) In high density cultures mrp1 is predominantly found on lateral membranes. No vesicular staining was apparent. N, nucleus.

Table 1. Inhibition of mrp1 increases sensitivity for certain oxidative stress inducing agents

| | Control (μM) | + MK571 (μM) |
|-------------------------------|-----------------|-----------------|
| Menadione | 6.3±0.8 | 2.3±1.6* |
| TBHQ | 59.8±12.0 | 20.5±8.1* |
| H ₂ O ₂ | 265.5±88.7 | 343.1±98.8 N.S. |

Hepatocytes isolated from normal rats were cultured for 3 days at low density to induce mrp1 expression. Thereafter cells were incubated with various concentrations of menadione (0-20 μM), TBHQ (0-300 μM) or hydrogen peroxide (0-800 μM), in the absence (Control) or presence (+MK571) of 25 μM of the MRP inhibitor MK571. After 20 hours, cell death was determined by MTT test as described in Materials and Methods. The concentrations at which 50% of the cells had died (LD50) was calculated for both conditions. Results are the mean ± s.d. of 3 separate isolations. Significant differences were determined using a two-sided paired Student's *t*-test.

**P*<0.05 compared to control. N.S., not significant.

protects hepatocytes that have entered the cell cycle against menadione and TBHQ toxicity but not against H₂O₂.

DISCUSSION

The substrate specificity of MRP1/mrp1 appears to be very similar, if not identical, to that of mrp2 (Müller et al., 1994; Leier et al., 1994; Jedlitschky et al., 1994). However, compared to mrp2, hepatic mrp1 expression is very low in normal liver tissue. To gain more insight in the regulation and the physiological function of mrp1 in hepatocytes, we studied the expression of mrp1 and mrp2 in relation to the cell cycle. As discussed before, the progression through the cell cycle was manipulated by culturing rat hepatocytes at different cell densities. The current study shows that hepatocytes cultured at high density do not express cyclin D1, which is normally expressed in late G₁, and do not show significant incorporation of BrdU as an indicator of DNA-synthesis. This is in line with results published by Loyer et al. (1996). These authors defined a restriction point (R-point) in late G₁ which hepatocytes, cultured at high density, cannot pass without stimulation by growth factors. Passing the R-point results in cyclin D1 expression and subsequent entry into the S-phase. We found that hepatocytes cultured at low density express cyclin D1 and display significant DNA-synthesis without adding growth factors. Thus, variation of cell density results in hepatocyte populations that are mainly in mid-G₁ (high density) or have progressed into the S-phase and beyond (low density).

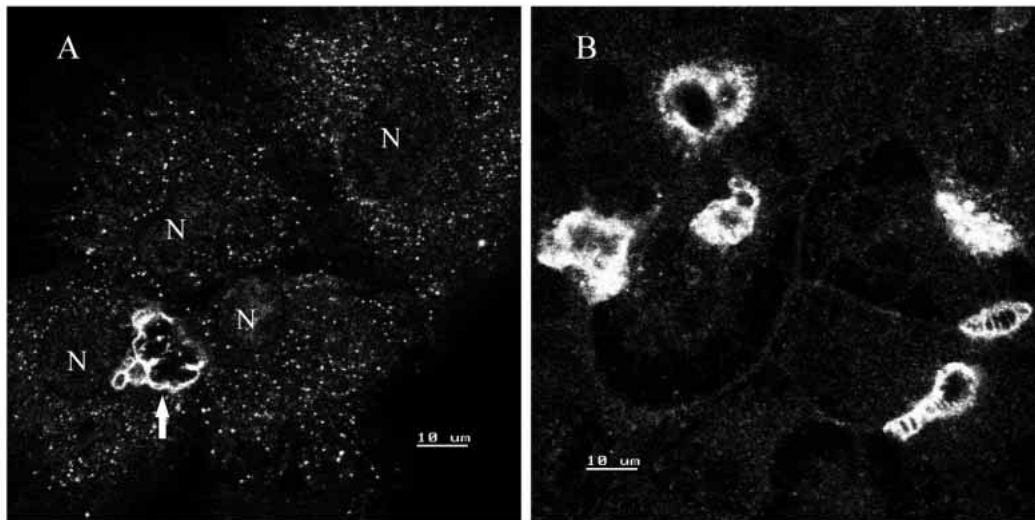


Fig. 6. Localization of mrp2 in low and high density cultures. Hepatocytes cultured for 24 hours at low and high density were stained for mrp2 with the polyclonal antibody MRPk4 followed by a secondary FITC-labeled antibody. Images were taken with a confocal scanning laser microscope. (A) In low density cultures mrp2 was predominantly found on intracellular vesicular structures. Occasionally, apical structures formed between two hepatocytes (see arrow) were positive for mrp2. (B) In high density cultures, mrp2 is mainly present in apical structures with little intracellular vesicular staining. N, nucleus.

Both in low and high density cultured hepatocytes dramatic changes in the expression of mrp1 and mrp2 were observed. In low density cultured cells, mrp1 mRNA expression is induced within 24 hours followed by an increase in protein levels. Concomitantly, mrp2 mRNA and protein levels rapidly decline. In high density cultured cells, a similar switch in expression from mrp2 to mrp1 is observed. However, mrp2 mRNA and protein persist longer in conjunction with a delayed up-regulation of mrp1 mRNA and protein synthesis. This delay reflects the slower progression through G₁ of hepatocytes cultured at high density. The switch from mrp2 to mrp1 expression takes place after the expression of c-myc but before cyclin D1 is expressed in the G₁ phase of the cell cycle. These data are in line with earlier reports in HL-60 leukemia cells and patients with neuroblastoma that describe a correlation between c-myc or N-myc and mrp1 expression (Norris et al., 1996; Akimaru et al., 1996; Ishikawa et al., 1994). In vivo, down-regulation of mrp2 has been found during endotoxemia, ethinylestradiol-induced cholestasis and cholestasis due to bile duct obstruction (Trauner et al., 1997). We recently demonstrated that during endotoxemia, mrp1 is induced while mrp2 is down-regulated (Vos et al., 1998). This indicates that the reciprocal change in mrp1 and mrp2 levels represents a physiological response of the hepatocyte.

The changes in mrp1 protein levels in TR⁻ cells, lacking mrp2, closely parallel changes in the transport rate of the GS-conjugate GS-MF across the plasma membrane (Fig. 4). This suggests that the increase in transport is mediated by mrp1. Therefore, mrp1 appears to be responsible for the stable level of GS-conjugate efflux observed in hepatocytes that have entered the cell cycle. However, it can't be ruled out that transporters other than mrp1 mediate part of the observed GS-MF efflux. To mediate this transport, one would expect mrp1 to be localized to the plasma membrane. This is indeed the case in high density cultured hepatocytes, in which mrp1 is present in lateral membranes. However, mrp1 is predominantly found

on the membranes of intracellular vesicles in low density cultured cells. Nevertheless, the GS-MF efflux in low density cultures is similar to that in high density cultures at 96 hours (Fig. 4). Since the expression level of mrp1 is also similar at this point, the only difference is the localization of mrp1 (intracellular vs plasma membrane). This indicates that intracellular vesicular mrp1 is somehow involved in GS-conjugate efflux. Possibly, substrate accumulates in mrp1-containing vesicles which then fuse with the plasma membrane, discard their contents and recycle back to the cytosol. Such a mechanism may be an efficient way to control cytosolic concentrations of potentially toxic compounds and has been proposed to explain GS-conjugate efflux in platinum-resistant HL-60 leukemia cells (Ishikawa et al., 1994). The numerous GS-MF accumulating vesicles observed in low density TR⁻ hepatocytes (Fig. 7) are consistent with this hypothesis. Also in HepG2 cells and SV40-immortalized human hepatocytes we previously observed that MRP1 was not detectable in the plasma membrane of cells in the absence of cell-cell contact (Schippers et al., 1997; Roelofs et al., 1997b). These data indicate that cell-cell contact is essential for a proper plasma membrane localization of MRP1. Vesicular mrp1 has been observed in normal tissues such as bronchiolar epithelium, keratinocytes and macrophages (Flens et al., 1996) and certain cell lines (Van Luyn et al., 1998; Ishikawa et al., 1994), suggesting that it may have a physiological function in these cell-types. Liver regeneration in vivo probably more resembles cell proliferation under high density conditions. Although under these conditions vesicular mrp1 seems less important, it may become important during stages in the cell cycle where cell-cell contact is transiently decreased.

Mrp2 expression appears to be associated with the existence of a well-defined apical domain. Previously it was shown that mrp2/cmoat-mediated transport across the plasma membrane rapidly decreases after isolation and culture of hepatocytes (Roelofs et al., 1995). This can be attributed to endocytosis of

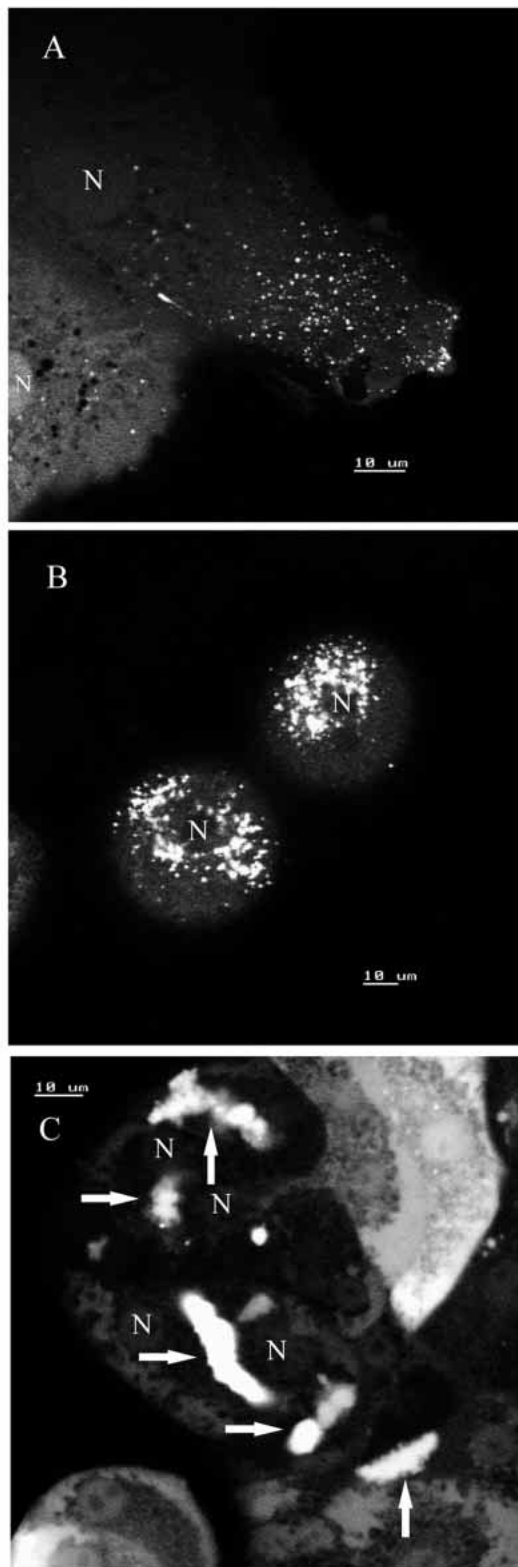


Fig. 7. Intracellular structures accumulate the fluorescent GS-conjugate GS-MF. Normal and TR⁻ hepatocytes grown on coverslips at low and high density were loaded with CMFDA at 10°C. Coverslips were transferred to fresh media and stored on ice. Intracellular accumulation of the MRP-substrate GS-MF was visualized by confocal microscopy. (A) In TR⁻ hepatocytes cultured for 72 hours at low density, GS-MF accumulates in numerous small intracellular vesicles. (B) In normal hepatocytes cultured for 24 hours at low density, GS-MF also accumulates in small intracellular vesicles. (C) In normal hepatocytes cultured for 24 hours at high density, GS-MF accumulates in large (apical) vacuoles. N, nucleus.

hours. This is stimulated by the cAMP analogue dibutyrylcAMP (Roelofsen et al., 1998). Other apical proteins such as the (canalicular) Cl⁻/HCO₃⁻ exchanger (Benedetti et al., 1994) and C-CAM 105 (Boyer and Soroka, 1995), also redistribute to the apical domain. Restoration of the apical domain does also occur in high density cultured cells, as is clear from the localization of mrp2 to apical vacuoles and the transport of substrate into these vacuoles (Figs 6 and 7). This is accompanied by a slower progression through G₁ and the persistence of mrp2 expression. In contrast, cells cultured at low density hardly formed apical domains due to diminished cell-cell contact. Under these conditions mrp2 is confined to intracellular vesicles and is more rapidly down-regulated. This suggests that the switch from mrp2 to mrp1 and a (partial) loss of cell polarity are associated events in G₁. In this respect it is interesting to note that c-Jun, expressed in early G₁, has been implicated both in the modulation of epithelial polarity (Fialka et al., 1996) and, as a component of the AP-1 transcription factor, in the regulation of MRP1 transcription (Zhu and Center, 1994).

The data presented in this paper indicate that GS-conjugate transport activity in hepatocytes that have entered the cell cycle is mediated by mrp1. We assessed the importance of mrp1 for the protection of hepatocytes in the G₁ phase of the cell cycle against agents that induce oxidative damage. By blocking mrp1 transport activity with a specific inhibitor, hepatocytes became 3-fold more sensitive towards menadione and TBHQ. Quinones form conjugates with GSH (Peters et al., 1996a; O'Brien, 1991; Akerboom et al., 1988) that remain cytotoxic (Peters et al., 1996b; O'Brien, 1991). Mrp1 probably protects by exporting these conjugates from the cell. The sensitivity of the cells towards H₂O₂ did not significantly alter by blocking of mrp1 activity. The main detoxification pathway for H₂O₂ is via the oxidation of GSH to GSSG. Although GSSG is a substrate for mrp1 (Leier et al., 1996), GSSG export from the cell apparently does not alter toxicity. Alternatively, extracellular H₂O₂ may cause cell death by causing extensive plasma membrane damage before it can react with intracellular GSH. Thus, mrp1 appears to be involved in the detoxification of oxidative stress-inducing quinones, but not of extracellularly administered H₂O₂.

In conclusion, our results indicate that in hepatocytes that have entered the cell cycle, mrp1 is induced while mrp2 is down-regulated. This switch in expression from an apical to a basolateral transporter with a similar substrate specificity occurs in mid-G₁ and appears associated with a decrease in cell polarity. Induction of mrp1 leads to preservation of GS-conjugate transport activity and protects cells that enter the S-phase against certain oxidative stress-inducing agents.

the canalicular domain as a consequence of the disruption of cell-cell contact during the isolation procedure. However, polarity is rapidly restored in short-time culture. In experiments with hepatocyte couplets, which consist of two hepatocytes forming an apical domain in-between, endocytosed mrp2 is redirected to the intact apical domain of the couplet within 3

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